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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE ANTIGENIC PROPERTIES OF *B. PSITTACOSIS.*

H. MARRIAN PERRY.

From the Pathological Department (Col. S. L. Cummins, C.B., C.M.G.) of the Royal Army Medical College, London.

Received for publication May 17th, 1920.

THE following investigation was undertaken to establish the identity of an organism isolated from a bird which sickened and died from an illness in which enteritis was a prominent symptom.

This bird, a grey touracou (*Schizorhis concolor*), belonging to a species native to South Africa, is entirely a fruit eater. It, together with six others of the same species, was imported into this country in December. A few weeks after arrival one died, and three others contracted, at short intervals, a fatal illness characterised by enteritis. Two only of the batch have survived. Post mortem, the intestines of the fatal cases were found to be congested and inflamed, and in one a well-marked peritonitis was present.

The bacillus, which was isolated from the heart-blood, spleen and intestinal contents, conformed in its morphology, staining and biochemical reactions to a member of the Salmonella group of organisms. It was a short bacillus with rounded ends, and was very actively motile. It was Gram negative. Cultures were readily obtainable on all media. It did not ferment lactose; glucose, mannite, and dulcite were fermented with the production of acid and gas. Indol was not formed.

Its pathogenicity was tested and it was found virulent for the following animals:

Mice.—Subcutaneous inoculation of 0·2 c.c. of a twenty-four-hour broth culture caused a septicæmia which proved fatal in 12 hours. The organism was recovered from the heart-blood, spleen, urine and intestinal contents.

Rabbits.—Intravenous inoculation of 0·3 c.c. of the same culture killed rabbits of 760 grm. weight in 48 hours. Positive cultures were obtainable from the heart-blood, spleen, bile, urine and intestinal contents.

Pigeons.—Feeding experiments were not successful. Intra-peritoneal inoculation of 0·2 c.c. killed a bird in 8 hours. The bacillus was recovered from the heart-blood.

The organism, considering its cultural reactions and source of origin, could be classified with the bacillus of psittacosis.

A short summary of the earlier work connected with this organism is of interest. The first human epidemic that can be attributed to it was described by Dujardin-Beaumetz (1) in 1892. A fatal form of infectious disease made its appearance amongst a consignment of parrots during transit to Paris from abroad. Three hundred birds died out of a total of 500. The surviving birds were distributed to various centres and human contact cases originated in the neighbourhood of many of them. In all 42 cases were reported in which the illness could be traced to this source of infection, and fourteen deaths occurred during the epidemic.

Dujardin-Beaumetz at first considered the disease to be of the nature of an infectious influenzal pneumonia, and whilst forming the opinion that it could have been spread from man to man through the intermediary of the parrots, he did not think it was a specific infection of these birds.

The following year Nocard (2) isolated an organism which he believed was the causal agent of this outbreak. As he did not have at his disposal any affected parrots he was obliged to limit his researches to the bone-marrow obtained from the wings of some of the birds that had died. He succeeded in isolating an organism, which he found not only pathogenic for parrots, but which also possessed a high degree of virulence for all laboratory animals.

Up to this time the organism had not been recovered from a human case of psittacosis, but this was accomplished later by Gilbert and Fournier (3) during the course of another small outbreak. They were able to isolate, both from the patients and from the organs of the diseased birds, a bacillus which corresponded with that described by Nocard.

B. psittacosis has, in view of its biochemical and cultural characteristics, been included in the Salmonella group of organisms. This group, including as its principal members *B. enteritidis* (Gaertner), *B. paratyphosus B*, and *B. aertrycke* (*suipestifer*), was rendered more complex by the admission of a number of other organisms obtained from various sources other than human, the more important of these being *B. typhi murium*, *B. danysz*, and *B. psitta-*

cosis. The work of Bainbridge (4) and others identified these latter bacilli with one or other of the principal members of the group, *B. psittacosis* being apparently identical with the strain of *B. aertrycke* employed in the comparison. However, it has recently been shown that considerable variation in antigenic properties exists between different strains of *B. aertrycke*, and that the serological differences brought into evidence by the absorption test are sufficient grounds for the recognition of various types of this organism. Schütze (5) (6), studying this question in detail, has recognised eight different types, the most common being a strain isolated from cases of food poisoning which is known as the "Mutton" type. According to this observer over 50 per cent. of "Aertrycke" bacilli, including organisms isolated from animal and human sources, are identical in antigenic properties with this strain. All of these are believed to be capable of causing an acute gastro-enteritis in man.

The object of this investigation was to determine (1) if this bacillus, definitely of avian origin, could be identified by its antigenic properties with one of the recognised types of *B. aertrycke*, and (2) what relationship it bore to the organism originally isolated by Nocard.

An agglutinating serum for the bacillus was prepared and its titre determined for the homologous organism *B. paratyphosus B*, and two types of *B. aertrycke*—"Mutton" and "Newport." Absorption tests were then made with each of these organisms, absorption with the homologous bacillus serving as a control of the technique employed. The results are given in the appended table.

Serum.	End point of agglutination titre for: Bacillus "X"	*B. aertrycke* ("Mutton")	*B. aertrycke* ("Newport")	*B. paratyphosus B.*
Bacillus "X"* . . .	25,000	25,000	4000	4000
Ditto absorbed with—				
1. Bacillus "X" .	< 500	< 500	< 500	< 500
2. *B. aertrycke*. ("Mutton") .	< 500	< 500	< 500	< 500
3. *B. aertrycke*. ("Newport") .	20,000	20,000	< 500	< 500
4. *B. paratyphosus B*	20,000	20,000	< 500	< 500

* The organism isolated from the bird is referred to in the above table as Bacillus "X."

It will be noted, on reference to these experiments, that the absorptive capacity of the Aertrycke "Mutton" strain corresponded with that of the organism under investigation. Both the specific and group agglutinins were removed from a serum prepared with this bacillus. Saturation with the Aertrycke "Newport" strain and *B. paratyphosus* left the specific agglutinins practically unaffected.

The converse experiment of saturating an Aertrycke "Mutton" serum with the unknown bacillus yielded the same result.

Serum.	End point of agglutination titre for: Bacillus "X"	*B. aertrycke* ("Mutton")
B. aertrycke ("Mutton")	25,000	20,000
Ditto absorbed with—		
1. *B. aertrycke* ("Mutton") . . .	< 500	< 500
2. Bacillus "X"	< 500	< 500

If the validity of deductions obtained by means of the absorption test be admitted, it would appear that the antigenic properties of the bacillus under examination and the "Mutton" type of *B. aertrycke* are identical.

Amongst the organisms studied by Schütze, in the investigation already referred to, a strain of *B. psittacosis*, which he states was "presumably of avian origin," was examined and was classified by him as of the "Mutton" type.

Through the courtesy of Dr. Besredka, opportunity was afforded me of comparing the organism under investigation with Nocard's original strain of *B. psittacosis*, a culture of which he kindly sent me. No difference in absorptive capacity was distinguishable between them, and in their antigenic properties they appeared to be identical.

Serum.	End point of agglutination titre for Bacillus "X."	*B. psittacosis.*
Bacillus "X"	25,000	25,000
Ditto absorbed with—		
1. Bacillus "X" . .	< 500	< 500
2. *B. psittacosis* . .	< 500	< 500

Whilst it would thus appear that no diversity, other than source of origin, exists between *B. psittacosis* and *B. aertrycke* of the "Mutton" type, it must be admitted that the question of permanency of these finer serological differences amongst members of this group of organisms has not been definitely settled. It may, however, be mentioned in its favour that no variation in this respect has been noted in strains of the organisms cultured for several years.

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A METHOD FOR THE ESTIMATION OF UREA BY SOY-BEAN.

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Received for publication May 19th, 1920.

NUMEROUS methods for the estimation of urea by means of soy-bean urease have been devised; the procedure described below is comparatively simple and gives remarkably accurate results with known amounts of urea. Some features of it to which attention may be directed are:

(1) The use of an alcoholic extract of serum without evaporation and re-solution in water.

(2) The direct titration of ammonia without removal by an air-current.

(3) The method of titration.

DESCRIPTION OF METHOD FOR SERUM.

(1) *Preliminary treatment of serum.*—5 c.c. of serum and 10 c.c. of methylated spirit are measured into a centrifuge tube; the estimation is, of course, carried out in duplicate wherever possible. The mixture is well stirred with a glass rod and centrifuged; 10 c.c. of the supernatant fluid are transferred to a 250 c.c. Erlenmeyer flask (if a very little of the precipitate is drawn up as well no appreciable error is caused) and 30 c.c. of water (from a cylinder) and 15 drops of 0·2 per cent. methyl red in alcohol are added. Sufficient 0·1 N. H_2SO_4 is added to produce a deep rose-red colour (about 1·5 c.c. will be required); the mixture is boiled for a few seconds to expel CO_2, cooled, and the flask stoppered if the subsequent neutralisation is not carried out at once.

(2) *Preparation of colour-standard.*—40 c.c. boiled water (*i.e.* the same volume as that in the Erlenmeyer neglecting the 0·1 N. H_2SO_4) and 15 drops methyl red are placed in a beaker and the indicator brought to the neutral colour (buff) by 0·01 N. H_2SO_4 or NaOH; to get the exact colour it is usually necessary to take fractions of a drop from the burette-nozzle on to the side of the beaker. One must select a particular shade of buff and adhere to it in the use of this method.

(3) *Method of colour comparison.*—A comparator of the very simple type used in C. J. Martin's method of titrating plasma is employed. A cardboard box, about 8 × 5 × 3 in., has the floor replaced by white paper and two holes are made in the longer side to admit two test-tubes; these must be of equal calibre, and are marked at the 10 c.c. level. The test-tubes are made to hang clear of the lower side of the box by means of rings of rubber tubing placed around them above the level of the holes in the upper side; 10 c.c. of the water neutralised to methyl red are poured into one of the two test-tubes, which is then corked. The acid fluid obtained as under (1) above is nearly neutralised by addition of 0·1 N. NaOH, the neutralisation being then carried on with 0·01 N. NaOH. When the neutral point is seen to be approaching, 10 c.c. of the fluid are poured into the other test-tube, and a comparison made with the standard. The fluid is subsequently poured back into the flask, but it is well to keep it in the test-tube until after the next addition of alkali to the flask, as a reserve of more acid fluid is then at hand in case the neutral point be overshot. Comparisons are made thus until an exact match is obtained, which with a little practice is brought about very quickly; the point of match is seldom in doubt over a range of more than one or two drops 0·01 N. This method of titration has been found very useful for other purposes, *e.g.* the titration of urine (see a forthcoming paper by Davies, Haldane and the writer in the *Journal of Physiology*). Colour-changes which seem very vague when observed in a flask in the ordinary way may be found to give a very sharp point when the box is used.

(4) *Decomposition of urea.*—2 grm. powdered soy-bean are placed in 100 c.c. normal saline; the mixture is allowed to stand with occasional shaking for ten minutes or so and then filtered. The extract can be kept for several days in the ice-chest. Two "blank" flasks are then prepared, each containing 40 c.c. boiled water and 15 drops methyl red; the water is then brought to the same reaction as the standard in the manner described above. 5 c.c. bean extract are then added to every flask, the extract being measured with as much accuracy as the serum and spirit.

The flasks are at once corked and incubated for one hour; a water-bath at 42° C. has been used as a rule in this work, but incubation in the air at 37° C. was found to be sufficient.

(5) *Titration of ammonia.*—15 c.c. 0·01 N. H_2SO_4 are added to each flask (a further quantity being added if the reaction is not then strongly acid). The liquid is then boiled for a few seconds, cooled, and the flask corked.

A fresh colour-standard is made, corresponding to the volume now present in the flasks, with 55 c.c. water and 15 drops methyl red. The excess of acid in the flasks is then titrated with 0·01 N. NaOH in the manner described under (3) above, the mean result of the blanks being, of course, subtracted. 1 c.c. 0·01 N. ammonia = 0·3 mgrm. urea.

TESTS OF THE METHOD.

Urea re-crystallised from alcohol was used. Kjeldahl estimations on it showed the following results:

0·1534 grm. gave 71·05 mgrm. N.; calculated, 71·58 mgrm.

0·0530 grm. gave 24·57 mgrm. N.; calculated, 24·73 mgrm.

A number of estimations were carried out on solutions of urea before the colour-comparator described above came to be employed; the results (Table I) were promising, but were irregular and sometimes higher than the calculated amounts. This was due, no doubt, to difficulty in determination of the exact end-point by the ordinary method of titration.

TABLE I.

Urea solution A, 0·1040 grm. in 200 c.c.:	C.c. 0·01 N. ammonia. Calculated.	Found.
10 c.c.	17·33	17·10, 17·25.
5 c.c.	8·66	8·70, 8·55, 8·50, 8·60, 8·40, 8·35, 8·70, 8·50.
3 c.c.	5·20	5·30.

When the comparator was used the results became more uniform (Table II); estimations were then made upon urea added to serum and cerebro-spinal fluid.

TABLE II.

Urea solution B, 0·0912 grm. in 200 c.c.:	C.c. 0·01 N. ammonia. Calculated.	Found.	
5 c.c.	7·60	7·50, 7·55, 7·55, 7·70.	
Urea solution C, 0·1472 grm. in 250 c.c.:			
5 c.c.	9·80	9·55, 9·45, 9·45, 9·45.	
5 c.c.; incubation in 25 per cent. spirit	—	9·45, 9·45 = 96·4 per cent.	
3 c.c.	5·88	5·75, 5·75, 5·65.	
3 c.c. added to alcoholic extract of serum	—	5·75, 5·75 = 97·7 per cent.	
2 c.c. added to cerebro-spinal fluid	3·92	3·87, 3·92.	
5 c.c. added to 10 c.c. serum (estimation by method as described for serum):			
(1) With serum I.	3·27 (= ⅗ of 5 c.c.)	3·15, 3·25, 3·20, 3·10	mean 3·173 = 97 per cent.
(2) With serum II	—	3·12, 3·22	

It may be well to give the details of one of the last experiments given in the table above.

Two mixtures, A and B, were made as follows:

	A	B
Serum	10 c.c.	10 c.c.
Spirit	30 c.c.	30 c.c.
Urea solution . .	5 c.c.	Water 5 c.c.

Two portions of 15 c.c. were taken from each supernatant fluid, added to 35 c.c. water, and the estimation carried out in the manner given at the beginning of this paper. The results were as follows:

	C.c. 0·01 N. ammonia.		
Blanks . .	1·20	1·25 mean 1·23;	this has been subtracted from results A and B below.
B . .	3·47	3·52 mean 3·50	
A . .	6·72	6·62	
Subtract B . .	3·50	3·50	
Urea recovered .	3·22	3·12 mean 3·17 = 0·951 mgrm. urea.	
Urea calculated .	3·27 = 0·981 mgrm. urea.		

NOTES ON METHOD.

The degree of accuracy of these results caused some surprise, as the technique described is, at first sight, open to numerous objections. Thus:

(1) No buffer solution is used, as in van Slyke's method, to prevent injury of the enzyme by the ammonia. Evidently the very minute amount present is not sufficient to have any such action; in an estimation on a normal serum as described above the concentration of ammonia would be less than 0·001 N.

(2) It might seem that the loss of ammonia which must occur when the flask is opened would be appreciable. 1 grm. of water at 42° C. and 760 mm. absorbs 0·29 grm. ammonia (Roscoe and Dittmar [1]); this concentration is nearly 20 N. The conditions of such a determination, in which the water is exposed to an atmosphere of pure ammonia, are, of course, very different from those of the method here described; but the result is quoted to show the very high solubility of the gas in water even at 42° C.

(3) In the final colour comparison the fluid titrated is turbid (owing to coagulated protein from the bean-extract and lipoid from the serum), while the standard is clear; but with a little practice the turbidity does not cause any serious difficulty.

(4) The 0·01 N. NaOH has been made, with boiled water, from a 0·1 N. solution prepared without any precautions against the presence of carbonate. But the results given in the table seem to show that the troublesome process of preparing CO_2-free alkali can in this case be dispensed with.

(5) The high "blank" value, which may amount to one-third of the total result, may seem undesirable, but the figures given in the table show that this does not affect the accuracy of the results. Duplicate blanks should not differ by more than 0·1 c.c. 0·01 N. The "blank" value is usually from 1·0 to 1·30 c.c. for 5 c.c. extract; it varies with different extracts and with the length of time that the extract is kept.

Other indicators were tried (neutral red, phenol red) but methyl red was found to give better end-points. The use of the alcoholic extract of serum, without evaporation, is a great advantage. Firstly, time is saved; secondly, if such an extract is evaporated to, or nearly to, dryness, a white residue is obtained which is not readily soluble in water, or water and alcohol, even on heating. When one attempts to neutralise this suspension, the results are very variable. No satisfactory results were obtained in this work until evaporation of the alcohol was abandoned. The results given in Table II show that even 25 per cent. spirit has no adverse effect upon the enzyme; while the proportion present in the method as described is not more than 14 per cent.*

Cerebro-spinal fluids have been examined by this method without any preliminary treatment with alcohol, from 0·4 to 10 c.c. of the fluid being added to 30 c.c. water. A fluid containing a large amount of protein could, of course, be treated like a serum. When an estimation is made on urine, the amount of urea present can be foreseen roughly from the specific gravity; 1 or 2 c.c. of a 1 in 10 or 1 in 20 dilution is then taken. In the selection of the most suitable amounts of serum or cerebro-spinal fluid for estimation there is bound to be difficulty with a substance of which the amount present may be anything from 10 to 800 mgrm. per cent.; these fluids may contain more urea than does a dilute urine. The method seems to give the most accurate results when from 3 to 6 c.c. 0·01 N. ammonia is produced. Estimations on 5 c.c. of uræmic cerebro-spinal fluid have been made in which 90 c.c. was obtained, but the agreement between duplicates is then not so close; in such a case one must use 0·1 N. acid after incubation. 5 c.c. of serum has been recommended in the account of the method because this is simple both for measurement and calculation, but smaller amounts even of normal serum are sufficient; thus if 3 c.c. of a serum containing 30 mgrm. urea per cent. were mixed with 12 c.c. spirit, and 10 c.c. taken from this, 2 c.c. 0·01 N. ammonia would be obtained, which could be estimated with accuracy sufficient for most purposes.

Serum has been used in this investigation because it is so much more easily obtained, but the method can be applied equally well to whole blood. Serum will, of course, contain a higher percentage of urea than the whole blood. Suppose a blood to contain 26 mgrm. of urea in 100 grm. and to have

* Between 30 and 40 per cent. of the theoretical amount of ammonia is produced from urea by bean-extract even in the presence of 80 per cent. of alcohol (2).

the composition given by Schmidt (3), namely

1000 grm. blood { 513 grm. corpuscles with 350 grm. water
487 grm. plasma with 439 grm. water

the red corpuscles being completely permeable to urea, 100 grm. of the serum of this blood will contain 29·6 mgrm. of urea. In view of the wide range of urea-content even in normal persons, it would seem then to be for most purposes unimportant whether serum or blood is used.

The following are some examples of results obtained by the method here described (Table III).

TABLE III.

	Urea per 100 c.c.
Serum, healthy person	38·5 mgrm.
Plasma, case of cyanosis	24 ,,
Pooled Wassermann sera A	34·5 ,,
,, B	29·8 ,,
,, C	27·5 ,,
Serum, nephritis of pregnancy	54 ,,
Ascitic fluid, peritoneal tumour	18 ,,
Œdema fluid, chronic nephritis	47·1 ,,
Cerebro-spinal fluid, uræmia—Case I: In life	153 ,,
,, ,, Case II: *Post-mortem*	543 ,,
Case, uncertain diagnosis, low protein intake { Serum	18 ,,
Case, uncertain diagnosis, low protein intake { Cerebro-spinal fluid	11 ,,

THE UREA CONTENT OF NORMAL BLOOD.

Some recent data on this matter, none of them obtained by hypobromite methods, are brought together in Table IV; no restriction of diet was enforced except as stated in the table. It appears that amounts of from 15 mgrm. up to 60 mgrm. per 100 c.c. may occur in persons considered to be perfectly

TABLE IV.—*Urea Content of Normal Blood.*

Subjects.	Urea, mgrm. per 100 c.c. blood.	Authors.
(1) 16 medical students and instructors, "strictly normal"	23·5 to 27·9 (per 100 grm.)	Folin and Denis (4).
(2) 17 patients "showing no disturbance of renal function," ages 14 to 58	16·5 to 50·3	Farr and Austin (5).
(3) 30 "normal men and women in robust health," ages 17 to 60, blood taken 3 hours after a standard meal	21·4 to 55·7	Gettler and Baker (6).
(4) 10 "perfectly normal adults," ages 20 to 40	21·4 to 49·3	McLean and Selling (7).

Subjects.	Urea, mgrm. per 100 c.c. blood.	Authors.
(5) "Young healthy adults," ages 20 to 35—		
(*a*) 28 persons on same diet	15·6 to 43·8	Addis and Watanabe (8)
(*b*) 106 estimations on 22 persons on unrestricted diet	22·5 to 60·0	
(*c*) 50 estimations on 1 person (A.) on unrestricted diet	24·5 to 60·0	
(*d*) 11 estimations on 1 person (M.) on unrestricted diet	27·0 to 51·0	
(6) 9 nurses and patients, "no subjects presenting evidence of metabolic disorder"	22·7 to 53·8 (one case, 64 to 85)	Hammett (9).

healthy. (One case in Hammett's series showed distinctly higher amounts: these are placed in brackets in the table.) It is noteworthy that the variations in a single person at different times are almost as great as those observed in a series of persons (Addis and Watanabe). Folin and Denis, in their early observations, seem to have happened upon a series in which the range was exceptionally narrow; the results of the five other investigations show fairly close agreement.

SUMMARY.

A technique for the estimation of urea by soy-bean is described by which amounts of 1 mgrm. can be estimated with an accuracy of 97 per cent. The ammonia produced is not removed by an air-current, and is titrated by means of a comparator. In the case of serum, an alcoholic extract is used without evaporation.

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THE VALUE OF THE VITAL-RED METHOD AS A CLINICAL MEANS FOR THE ESTIMATION OF THE VOLUME OF THE BLOOD.

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MANY methods have been devised for the indirect determination of blood-volume, but few, unfortunately, can be applied to man. With the exception of those methods involving the inhalation of gases, they depend on the introduction of a known quantity of a foreign substance, *e.g.* a dye, into the blood-stream and the estimation of its dilution by the blood after a certain time. Time is allowed for mixing, but not long enough to permit appreciable escape of the dye from the circulation. From the standpoint of safety and simplicity vital red is probably the best substance which has been used so far for this purpose. This method is due to Keith, Rowntree and Geraghty, in whose paper (1) may also be found a good discussion of the older methods. The blood-volume was determined in this way by Keith (2) in cases of wound shock. The method yields values which are higher than those obtained by older methods, yet notwithstanding this, the results in wound shock (2) were found to be below the normal of the standard of even the old methods, thus emphasising the great drop in the volume of the circulating fluid in secondary shock.

The following investigation, suggested to me by Professor Starling, was carried out to test the reliability of the vital-red method for the clinical estimation of blood-volume.

The factors which are likely to disturb the absolute value of the result obtained by this means were studied under the following heads: (i) the uniformity of the dissemination of the dye throughout the whole of the circulating fluid, and the time taken for dissemination; (ii) the degree of the retention of dye by the plasma to the exclusion of the cells of the blood; (iii) the disappearance of the dye from the circulating fluid.

The animals used were cats and dogs; the latter possess the double advantage of a larger body-weight and a relative volume of plasma to corpuscles similar to that in man (57 per cent.: 43 per cent.).

Anæsthesia was induced by inhalation of a mixture of equal parts of chloroform and ether; to minimise the hæmolytic effect of the chloroform and ether, the maximum of analgesia was attained by a large dose of morphia given hypodermically about ten minutes before the chloroform and ether.

TECHNIQUE OF THE VITAL-RED METHOD.

(i) An initial sample (10 c.c.) of blood is drawn from a large vein into a paraffined centrifuge tube dusted inside with finely powdered potassium oxalate. This provides plasma for making up the standard for colorimetry and a sample of blood for the hæmatocrite.

(ii) A measured volume (0·2 c.c./1 kilo) of a 1·5 per cent. aqueous solution of vital red in a syringe is slowly (1 minute) introduced into a large vein. This may be effected by vene-puncture, or by inserting the needle into the rubber tubing connecting a burette to a cannula in the vein and then washing the dye in with gum-saline from the burette. Both methods were employed; the latter was found more convenient in cats.

(iii) Time is allowed for the dye to mingle with the circulating fluid (3 to 5 minutes, Keith) and a sample (10 c.c.) of dyed blood is drawn from a different vein into a pipette fitted with a venepuncture needle, which has previously been rinsed out with 1·6 per cent. sodium oxalate (3) to prevent clotting and hæmolysis; the blood is delivered into a prepared centrifuge tube. When repeated samples, *e.g.* every half minute, are required a cannula is inserted into the distal end of a vein or the proximal end of an artery, the first part of each sample being rejected.

The centrifuge tubes containing the blood are inverted half a dozen times so as to diffuse the oxalate for the prevention of clotting. They are then centrifuged at high speed (3000 revs./min.) for 20 minutes.

Some of the initial blood (i) having been drawn into the hæmatocrite tubes these are centrifuged at the highest possible speed for 2 to 3 minutes, until the corpuscles form a perfectly translucent mass.

(iv) A dilute solution of the dye is made up by diluting 1 c.c. (measured in a standard pipette) of the injected solution to 200 c.c. with saline in a graduated flask.

A small pipette of about 2 to 3 c.c. capacity is made, unit volume is etched on it, and it is fitted with a rubber teat. The following solutions are then made for comparison with the Dubosq colorimeter:

The standard.		Sample after injection of dye.	
Plasma	1 vol.	Dyed plasma	1 vol.
Saline	1 „	Saline	3 vols.
Diluted dye solution ($\frac{1}{200}$)	1 „		
Saline	1 „		

Let r = ratio of strength of dye in sample to that of the standard,

then $r \times \frac{1}{200}$ = strength of dye solution in sample.

If v = vol. of dye solution injected,

and x = vol. of plasma,

then $\frac{v}{x} = \frac{r}{200}$,

$$\therefore x = \frac{200 \times v}{r}.$$

Thus—

$$\text{volume of plasma} = \frac{\text{dilution factor} \times \text{no. of c.c. dye injected}}{\text{ratio of strength of dye in plasma}}$$

also—

$$\text{volume of blood} = \text{vol. of plasma} \times \frac{100 \text{ per cent.}}{\text{per cent. plasma by hæmatocrite}}$$

and—

$$\text{weight of blood} = \text{volume of blood} \times \text{sp. g.}$$

TECHNIQUE OF THE CONTROL METHOD.

The vital-red method was checked by methods involving either the reduction of the total blood by a known volume by bleeding or by increasing the circulating fluid by the intravenous infusion of gum-saline (Bayliss' fluid). The old method of bleeding and washing out the vessels was abandoned on account of the uncertainty of opening up all the stagnant capillary areas which one meets too often in histological technique; the experiments performed by this method showed the result to be only slightly below that obtained by the method about to be described:

Comparison.	Cat. 1.	Dogs: Average.
Bleeding and washing out blood-vessels . . .	168 c.c. .	(Two) 6·8 per cent. body-weight.
Hæmorrhage to the limit of existence . . .	175 c.c. .	(Six) 7·0 per cent. body-weight.

An initial sample of blood is taken for the colorimetric standard; 1 c.c. is carefully measured in a pipette and introduced into 199 c.c. of distilled water in a flask, coal-gas is bubbled through and the flask is tightly corked. The animal is now bled from a cannula placed in the femoral artery into a graduated cylinder containing oxalate, whilst warm gum-saline from a burette is introduced at the same rate into the external jugular vein. By this means quite nine-tenths of the hæmoglobin can be removed before the animal dies. When the animal shows signs of failure, the bleeding and gum-saline infusion are arrested. Time (3 to 5 minutes) is allowed for the thorough mixing of the remaining blood and gum-saline, and then a sample (referred to as the

"ante-mortem sample") is taken for colour comparison, enabling one to estimate the hæmoglobin left in the vessels. By the infusion of gum-saline at the same rate and in the same amount as the blood withdrawn the volume of the circulating fluid is kept practically constant (4).

Records of the arterial blood-pressure in the carotid showed the blood-pressure to be maintained at a constant level under these conditions, notwithstanding the enormous dilution of the hæmoglobin.

The initial sample, the mixed drawn blood (oxalated) and the last ante-mortem sample are compared as carboxy-hæmoglobin in the Dubosq colorimeter. The blood-volume can then be calculated:

If s = c.c. in first sample and 100 per cent. = per cent. Hb. in s;
b = „ by bleeding and β = per cent. Hb. in b;
and a = per cent. Hb. in the last ante-mortem sample; then—
$100-a$ = per cent. Hb. lost by the animal during bleeding.

$$\therefore \text{ total volume of blood} = \left(s + b\,\frac{\beta}{100}\right) \times \frac{100}{100 - a}.$$

The following example illustrates this:

Dog 2.—i. Initial sample . . 1 c.c. . . . 100 per cent. Hb.
ii. Hæmorrhage . 1350 „ . . . 37 „ Hb.
iii. A-M sample . . 1 „ . . . 9 „ Hb.

$\therefore$ (i) + (ii) = 91 per cent. of the blood $\equiv$ 1 c.c. @ 100 per cent. Hb.
+ 1350 c.c. @ 37 per cent. Hb.
$\equiv$ (1 + 499) c.c. @ 100 per cent. Hb.
$\equiv$ 500 c.c.

$$\therefore \text{ 100 per cent. of the blood} = 500 \times \frac{100}{91} = 550 \text{ c.c.}$$

The dilution of corpuscles was also used as a check on the Hb. method in those cases where there was no trace of hæmolysis; the agreement was found to be a very close one:

Blood-volume (weight) expressed as a percentage of body-weight.

By Hb. dilution . . . = 7·04 per cent. (average for 6 dogs).
„ corpuscular dilution = 7·00 „ „ „ 9 „

This method of maximal dilution of the blood *in vivo* yields, in the writer's opinion, the nearest approach hitherto attained to the absolute value of the blood-volume, and is therefore used as a basis of comparison for the other methods, a factor being determined in each of the other methods which is necessary to convert the observed result into the true one. Even measurements of the concentration of dye in the circulation may yield, under certain experimental conditions in animals, values much nearer the true than the usual vital-red technique. Thus, simultaneous bleeding and accurate replace-

ment of fluid by infusion of gum-saline in about one hour's time after the injection of large doses of dye gave the following results:

	Dye.	Blood withdrawn. = gum saline infused.	Blood-weight.	
			Calculated from dilution of dye.	Bleeding method.
Dog 9	Vital-red 30 mgrm./kilo.	160 c.c. 400 c.c.	6·77 per cent. 6·65 per cent.	6·43 per cent.
Dog 10	Congo-red 20 mgrm./kilo.	300 c.c.	7·3 per cent.	7·2 per cent.

DISTURBING FACTORS IN THE VITAL-RED TECHNIQUE.

(i) *The optimum interval for withdrawal of blood* after injection of the dye was found to be limited on the one hand by the blood-flow and on the other hand by the disappearance of the dye from the circulation. When the anæsthesia was deep the blood-volume yielded a low figure, indicating a small circulation for the dye, probably because some capillary areas were closed to the dye. On applying massage and passive movements the 4-minute interval yielded a much higher value, as shown by the following two cats of nearly equal weight—3·8 and 3·75 kilos.:

	Blood-volume as percentage of body-weight.	
	Vital red.	Bleeding.
Cat 1	6·0 per cent.	4·77
Cat 2—massage and movement	6·6 „	4·85
Difference . . .	0·60	0·08

The rate of the dissemination of the dye in the blood-stream was determined by injecting the dye into the proximal end of the left external jugular vein and withdrawing samples of blood every half minute from the distal end of the right external jugular vein. Curve 1 shows that the dye reaches its maximum concentration in the general circulation in the third half minute; interpolation gives it as eighty seconds—a period which is about twice the total circulation time (5) for a dog of this size (9¼ kilos.). Probably twice the total circulation time is long enough for thorough admixture of the dye in man, in which case 2–2½ minutes would be the optimum time for the withdrawal of blood. The way in which the time of withdrawal after injection of the vital red affects the result is shown in—

Dog 13 | 9¼ kilos., 8 mgrm. dye/kilo., 1 *or* 1½ *minute sample.*

Blood-volume (weight) as a percentage of body-weight:

Vital-red method = 8·8 per cent.

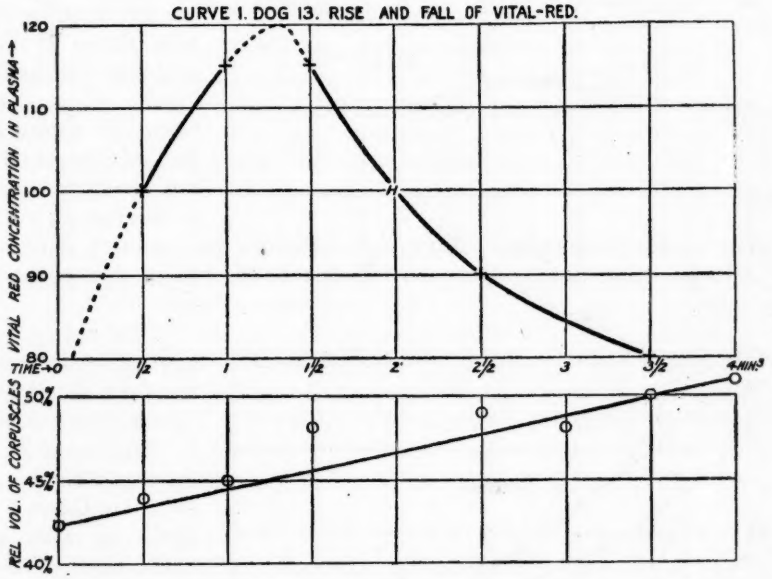
Bleeding . . = 7·9 „

which give the factor for correcting the vital-red result $= \frac{7{\cdot}9}{8{\cdot}8} = {\cdot}90.$

Whereas—

the average conversion factor for six other dogs on a 4-*minute* sample = ·78.

This experiment shows an interesting rise in the relative corpuscular volume with hæmorrhage. Another experiment was performed to determine whether this was an actual concentration of corpuscles or was merely an imbibition of fluid by the corpuscles from the plasma.

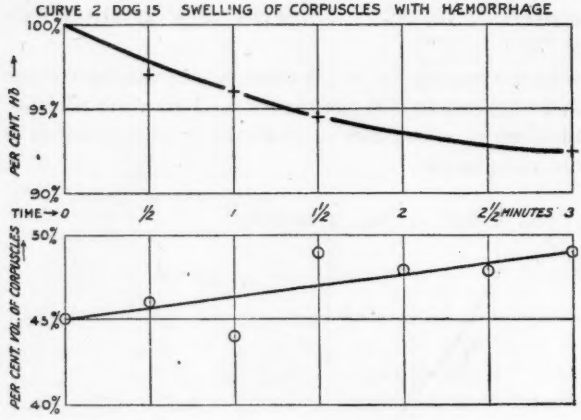


The gradual fall in percentage of hæmoglobin in spite of the rise in the percentage volume of corpuscles proves the swelling of the corpuscles as seen in Curve 2.

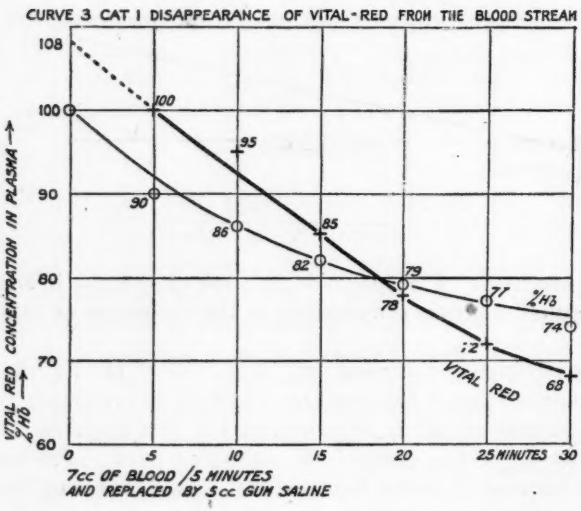
This may be a local phenomenon occurring only at the site of the hæmorrhage where there is a sudden drop in the pressure to which the corpuscle is exposed, or it may be a general condition, representing the first stage in the rapid withdrawal of fluid from the tissues. It may also be a defensive mechanism to minimise the loss of blood from the wound by increasing the viscosity of the blood.

Hooper, Smith, Belt and Whipple (3), using brilliant vital red, recently found that the concentration of this dye at the end of 2 minutes and 4 minutes was practically the same (100 : 99·9), and they state that the "blood-volume

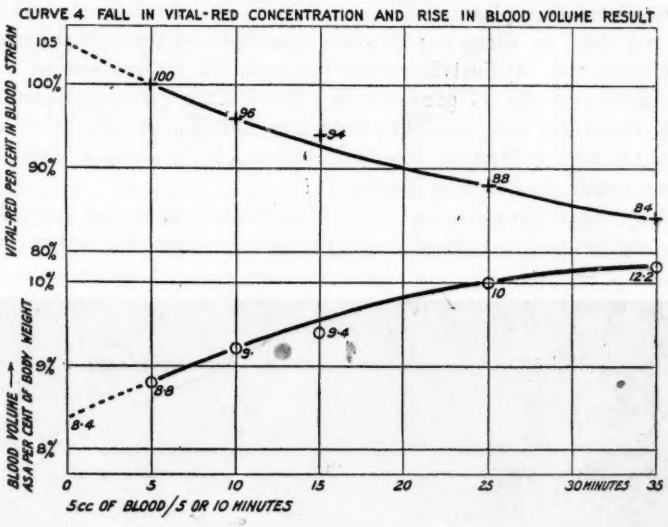
estimations based on samples collected at the 4-minute interval cannot be invalidated because of the elimination of dye from circulation." The equality of these concentrations may equally well be accounted for by leakage from the beginning, their first point (100) being on the up curve and their second point (99·9) on the down curve (compare Curve 1); it is probable that the elimination of any non-toxic dye varies with its concentration in the blood-stream, and is therefore at a maximum at the very beginning. Their high result for the amount of blood in dogs (10 per cent. body-weight) may be due to the loss of dye in the first 4 minutes.



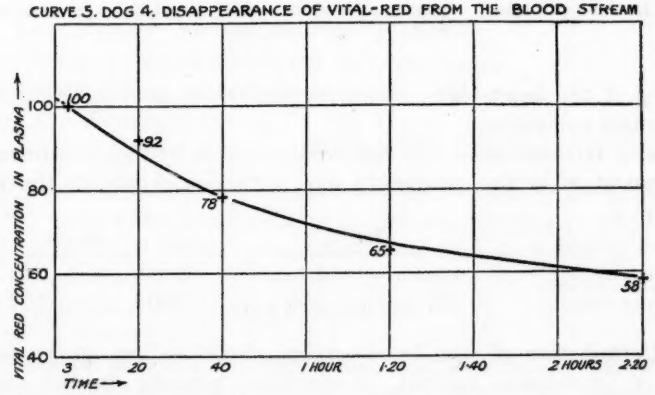
The rate of disappearance of vital red from the circulation is seen in Curves 3, 4 and 5. The loss of fluid in the case of this smaller animal in Curve 3 was made up, after each withdrawal of blood, by the intravenous infusion of an equal volume of gum-saline. The repeated small hæmorrhages lower the concentration of the dye at a faster rate than the fall in percentage of Hb., proving conclusively that the dye leaves the circulating plasma. The way in which this leakage vitiates the results of the calculated blood-volume is shown in Curve 4, in which the dog makes up the loss of fluid by drawing on his tissues. The result is seen to rise



steadily as time passes, and the value obtained by extrapolation (8·35 per cent.) is no doubt nearer the true blood-volume than the value (8·8 per cent. of the



body-weight) given by the usual vital-red technique. The percentage of hæmoglobin was estimated both as carboxy-hæmoglobin and as acid-hæmatin; the purity and depth of colour in the latter method was found to be more than

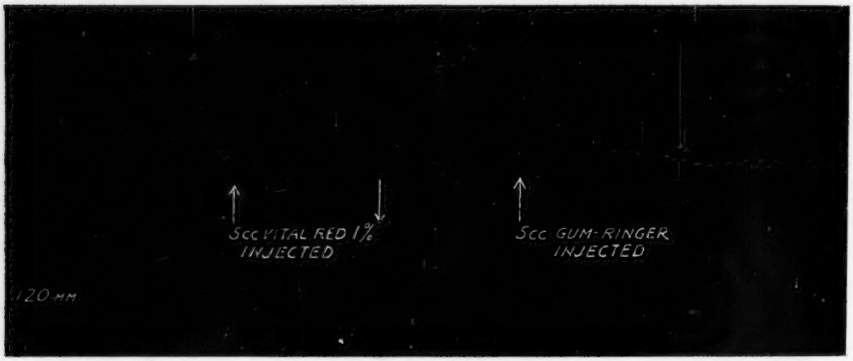


counterbalanced by the tendency of the acid-hæmatin particles to aggregation and precipitation. The carboxy-hæmoglobin still remains, as Boycott has shown, the best method for the colorimetric estimation of hæmoglobin,

especially in the comparison of a large number of samples of very different dilutions.

In Curve 5 the fall was observed at longer intervals, the concentration of dye in the circulating plasma falling to 72 per cent. in 1 hour. The sample of vital red used in these experiments was obtained through the courtesy of Dr. Dale from the Medical Research Council. A comparison of the above curves with the results (77 per cent. in 1 hour) of the two experiments quoted by Keith, Rowntree and Geraghty show the colloidal properties of the vital red prepared by the Medical Research Council to be only slightly different from the German dye used by Keith.

Tracings taken during some of the experiments show that vital red causes only the slight and transient physiological effects—rise of blood-pressure and



TRACING 1.—Cat 1 (3·8 kilos.). Tracing showing temporary rise of blood-pressure caused by intravenous injection of vital red.

quickening of the heart-beat—caused by the intravenous injection of almost any innocuous substance.

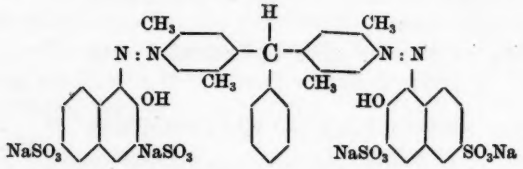
Repeated determinations are rendered valueless by the curvilinear fall in the concentration of the circulating dye. This is shown in the following estimations:

	Dog 1.		Dog 4.	
Time . . .	4 min.	1 hr.	4 min.	3 hr. 40 min.
Plasma volume .	237 c.c.	296 c.c.	200 c.c.	277 c.c.

Re-determination of the blood-volume, therefore, cannot be performed while any appreciable amount of the dye remains in the circulation. Smith (6) recently described a method for repeated determinations in the same animal at short intervals in which the error does not exceed 5 per cent.

(ii) *The degree of the retention of the dye by the plasma to the exclusion of*

the cells of the blood.—Vital red is disodium disulphonaphthol-azo-tetramethyl-



triphenyl-methane, hence the colloidal particles of the acid dye should carry negative charges. This was verified by kataphoresis in Hardy's (7) modification of Whetham's apparatus when vital red was found to dissociate so that the coloured particles moved to the anode. Now the charge on hæmoglobin is electro-positive, suggesting an affinity for the dye. A preliminary experiment was performed to determine whether the intact corpuscles also carried a positive charge. On examination in an electric field by a method described elsewhere (8) the corpuscles in freshly drawn blood were found to carry the same charge as the contained hæmoglobin.*

Experiments were then performed to determine whether the electro-negative dye was adsorbed to any appreciable extent by the electro-positive corpuscles.

Experiment 1—on fresh oxalated dog's blood:

(i) Determine the relative volume of corpuscles and plasma with the hæmatocrite.

Let p = percentage of plasma.

(ii) Place $\left\{\begin{matrix}24 \text{ c.c. whole blood} \\ 1 \text{ c.c. vital red solution}\end{matrix}\right\}$ in a centrifuge tube.

Invert gently and repeatedly for 5 minutes.

Centrifuge for 20 minutes at 3000 revs./min.

Pipette off the stained plasma.

Then dilution of dye if in plasma *only* $= \dfrac{100}{1 + \cdot 24\, p}$ per cent. (*by calculation*).

(iii) Centrifuge 25 c.c. of whole blood and pipette off the plasma. To 9 c.c. plasma add 1 c.c. of the vital red solution. Then dilution of dye = 10 per cent., and this is used as a standard of comparison for the actual strength of dye in the plasma from (ii), by means of the Dubosq colorimeter.

* Since the above was written Prof. Bayliss has found that corpuscles suspended in isotonic sugar solution, when examined in bulk in Whetham's apparatus, carry negative charges, *i. e.* opposite to what was found microscopically in whole blood in contact with glass (7). This brings the corpuscular envelopes into line with other tissue cells and renders intelligible the impermeability to the electro-negative dye vital red as well as the Hamburger effect.

Results:

Taking the strength found by calculation for (ii) as	100	per cent.
The colorimeter gave the actual dye in plasma of (ii) as	98·4	,,
Hence: dye taken up by corpuscles =	1·6	,,

(A value within the limits of error in colorimetric comparisons.)

Experiment 2.—To 10 c.c. of blood add 5 c.c. dye solution.

Centrifuge at high speed for 40 minutes.

The layer of white corpuscles resting on the erythrocytes appeared deeply stained, yet on microscopical examination neither the nucleus nor protoplasm was found to contain the dye.

As similar results were found by Keith (1) it was not considered necessary to carry out any further experiments in this direction.

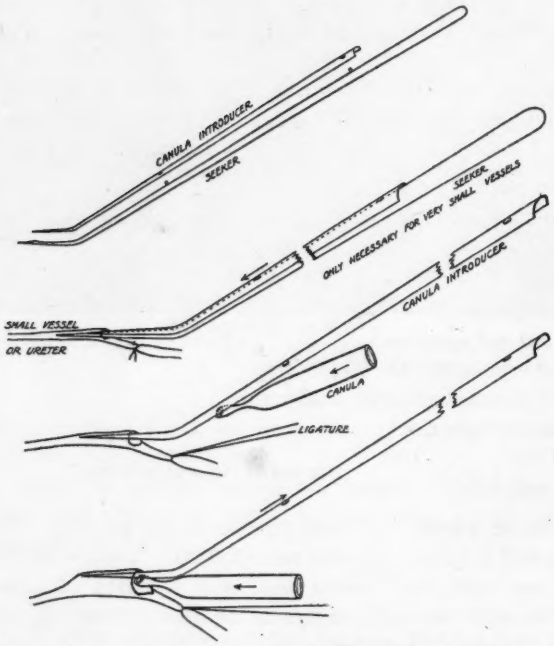


FIG. 1.—Cannula Introducer. (To be obtained from A. E. Braid & Co., Ltd., 30, Gower Place, London, W.C. 1.)

(iii) *Elimination of the dye.*—For a study of the leakage of vital red from the circulating plasma, lymph and urine were collected during and after the injection of the dye. To facilitate the introduction of cannulæ into the thoracic duct and into the ureters of small as well as large animals a cannula introducer (Fig. 1) was devised.

Dogs only were used in this group of experiments; they were placed under chloroform and ether anæsthesia after a preliminary dose of morphia. A cannula was placed in the thoracic duct and in each ureter, the ureters being exposed extra-peritoneally.

About ten times the usual clinical dose (3 mgrm./kilo.) of vital red was injected into the external jugular vein. The dye appeared in the ureter first

(five minutes), later in the thoracic duct (ten minutes), the difference being accounted for by the longer passage presented to the dye in the latter case after leaving the blood-vessels. In order to increase the lymph and urinary flow warm gum-saline was introduced into the same vein.

At the end of an hour a comparison of the strengths of dye was made as shown in the following results:

		Colorimeter.	Concentration.
Plasma	At 5 minutes	100 per cent.	
	At 1 hour	50 „	
	Average .	(75) . .	1
Lymph . . .		89 per cent.	1·2
Urine . . .		356 „	4·7

(The high concentration of colouring matter in the urine may be explained by the presence of urinary pigments or by the resorption of fluid by the tubules.)

The rapid fall in the concentration of the dye in the circulating plasma—to one-half in one hour—is accounted for by the unusual supply of saline in this case by intravenous infusion for the promotion of an active flow of lymph. Under ordinary conditions the dye is not eliminated at half this rate (Table on p. 157) and is not so obvious in the bladder urine, the saline acting as the diuretic in the above experiment. The urine was found to obtain albumen in this experiment. Probably the best time for making a determination of the blood volume would be immediately before lunch, thus minimising the error due to elimination of the dye with the digestive secretions and urinary excretion.

COMPARISON OF METHODS.

Reference to the experimental results (on p. 154) shows that for the vital-red method it is necessary to multiply the result obtained by the dye method when taking a 4-minute sample—

in the case of	cats	by 0·78 (average of 2 cats).
	dogs . . .	by 0·78 (average of 4 dogs).

As the factor appears to be identical for the two different species it is suggested that the same factor might be available for the correction of the very interesting figures published by Keith (1) of the blood-volume of man in various pathological conditions. Before this can be accurately determined it will be necessary to make due allowance for the slower circulation time in man on the basis of a 4-minute sample of vital-red plasma. As a first approximation, 0·8 may be taken as the correction factor, thus:

Average blood weight in man by the vital-red method (Keith, etc.) = 8·8 per cent. body-weight;

$$\text{Corrected value} \triangleq 8{\cdot}8 \times 0{\cdot}8$$
$$\triangleq 7 \text{ per cent. body-weight.}$$

It is interesting to note that in the two criminals who were bled to death

Bischoff found 7·6 per cent. as the average (the accepted standard 1858–1881) which is a little higher than the above value, probably because he washed out some myo-hæmatin from the muscles. Further, the corrected value (7 per cent.) occupies a mean position between the value (5 per cent.) obtained by the CO-method in the hands of Haldane and Lorrain Smith (9) and the vital-red method (8·8 per cent.) by Keith, etc. The low figure yielded by the CO inhalation and carmine titration method may be accounted for by the direct toxic action of CO on the vessel walls thus shutting off large capillary areas where circulation is at a standstill; moreover physiological substances other than hæmoglobin may show an affinity for CO (Barcroft).

Table showing the Results of Different Methods of estimating Blood-volume.

No.	Weight in kilos.	Vital red mgrm./kilo.	Blood volume in c.c.: Bleeding to death.	Blood volume in c.c.: Hb. method.	Blood volume in c.c.: Vital-red method.	Blood = per cent. body-weight: True value.	Blood = per cent. body-weight: Vital red.	Correction factor = true/dye.
Cats.								
1	3·8	12	168	175	217	4·8	6·0	0·80
2	3·75	3	174	—	235	4·86	6·6 m	0·76
3	3·5	3	—	158	h	4·66	h	—
7	2·6	6	—	111	h	4·48	h	—
(m = massage and movement.)								
					Average (cats)	4·7 %	6·3 %	0·78
Dogs.								
1	6	3	395	—	530	6·98	9·39	0·75
2	8·75	3	550	—	690	6·65	8·37	0·79
3	8·5	17½	—	542	702	6·79	8·78	0·77
4	5	3	—	357	h	7·57	h	—
7	6	2½	—	408	h	7·13	h	—
8	9¾	2	—	682	844	7·38	9·20	0·80
By the vital-red method (4-minute sample)—average (4 dogs)							8·9%	0·78
*9	5	30	—	304	628	6·43	*13·3	∴ very large dose
18	9¼	8	—	690	765	7·9	8·8	0·90
							1-minute sample	
By bleeding—average (8 dogs)						7·1%		

* 6·7 per cent. from dilution of dye by hæmorrhage. (h = hæmolysis)

FURTHER STUDIES OF THE INDIRECT METHOD.

Hæmolysis, in however slight a degree, renders the vital-red test valueless —a complication more often met with in cats than in dogs. The ideal dye would be one, therefore, which would circulate in a form which has a reddish tint, thus giving rise to no peculiar colouring of the lips of the patient, and yet, after withdrawal from the circulation, could be converted chemically into a deep colour not to be confused with hæmoglobin. Congo red, which in the form of the sodium salt is red and in the form of the free acid is blue, was thought to fulfil these requirements. On putting this to the test of experiment

it was found that on acidification of the dyed plasma the small dose of dye permissible in clinical cases was far too feeble for comparison even in the Dubosq colorimeter when used in this way. For checking the results in animals, dyes of various colours were tried, the methods of study being indicated below.

Electro-negative dyes.—As an example Congo-blue may be quoted. A 1·5 per cent. solution in distilled water was made and sterilised for 15 minutes at boiling-point. It was filtered through glass-wool whilst hot and twice again when cold. The solution showed no sedimentation after 24 hours.

A mixture of 1 c.c. 1·5 per cent. dye + 24 c.c. blood was put in a centrifuge tube and gently inverted periodically for 5 minutes. On centrifuging for 20 minutes at 3000 revs./min., although the corpuscles took up none of the dye (as one would expect from the positive charge on the corpuscles) yet hardly any of the dye remained suspended in the plasma. Thus Congo-blue, though possessing desirable indiffusibility, yet in solution occurs as colloidal aggregates too large for our purpose. Dawson, Evans and Whipple (10) have recently tested this dye in dogs and found very low readings in the plasma.

Electro-positive dyes.—Night-blue may be taken as an example. The solution was prepared as above. 1 c.c. of ¾ per cent. solution was injected into a dog; a sample of blood withdrawn 5 minutes later showed only a barely appreciable quantity of the dye. This electro-positive dye was probably adsorbed by the electro-negative tissues.

Other solvents.—The very complete indiffusibility of aniline blue was a clear recommendation for this dye, but the difficulty of obtaining the variety which is soluble in water prevented its use in the same way as vital red. When dissolved in ether, and though introduced into the circulation very slowly, yet its toxicity soon revealed itself. Weaker solutions were then tried, but the samples of plasma yielded were not found deep enough in colour for accurate comparison.

Histological dyes.—Of these, pyronin was found to be one of the best, but even this was inferior to vital red. They are mostly too diffusible.

Dawson, Evans and Whipple (10) have recently shown that a very large number of dyes possess the properties essential for determination of blood-volume. They find that a blue azo dye, T 1824 (ortho-toluidin with 2 molecules 1–8-amido 2–4-disulphonic acid), is slightly superior to vital red, especially as regards ease and accuracy of colorimetric readings.

Emulsions.—Various dyes were worked into a fine emulsion with petroleum but were found totally impracticable on account of viscosity and embolism, though the perfect indiffusibility of petroleum has much to commend it.

Suspensions.—Indian ink was evaporated over a water-bath to drive off the toxic terpenes. It was then worked into a fine paste with water and extracted several times. It was sterilised, filtered thrice through glass wool, and finally centrifuged to precipitate the coarser particles. 10 c.c. of the supernatant fluid

were injected into a dog but hardly a trace was found 5 minutes later in the circulating blood; it may have undergone phagocytosis in the liver, etc.

Of all the dyes tested Congo-red seemed to possess most of the desired properties, and a fuller investigation of this dye was undertaken. It is a non-toxic, comparatively indiffusible dye to living tissues, and it has been shown by Prof. Bayliss to consist mainly of single molecules (11), and so does not sediment in the centrifuge tube. It possesses the great advantage over vital red that when the dyed plasma is being compared with the standard in the Dubosq colorimeter, the addition of a drop of ammonia to each clears up the fluid and brightens the Congo-red, thus making the comparison easier to the eye. The technique in all other respects is similar to that of the vital-red method. The following are the results obtained:

Cats.

No.	Weight in kilos.	Congo-red mgrm./kilo.	Blood-volume in c.c. Bleeding.	Blood-volume in c.c. Congo-red.	Blood = per cent. body-weight. Bleeding.	Blood = per cent. body-weight. Congo-red.	Conversion factor = true/dye.
4	3·0	17	133	156	4·66	5·49	0·85
5	1·75	8	—	92	—	5·50	—
6	3·0	5	—	156	—	5·49	—
					Average (3 cats)	5·5 per cent.	

Dogs.

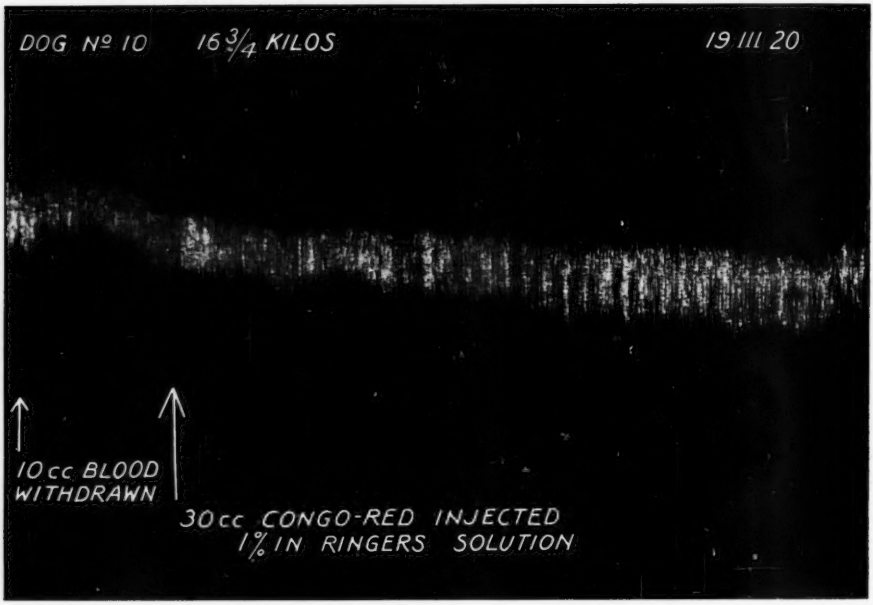
5	10·0	1½	—	662	—	7·02	—
6	7·7	5	485	540	6·69	7·42	0·90
11	4·75	10	303	333	6·76	7·42	0·91
12	9·75	10	670	750	7·30	8·15	0·89
14	8·9	5	525	615	6·25	7·32	0·85
15	16·5	3	1200	1255	7·7	8·06	0·96
					By the Congo-red method—average	7·6%	×0·9
*10	16·75	20	1136	1750	7·2	*11·7	∴ very large dose.
					By bleeding—average	7·0%	

* 7·3 per cent. from dilution of dye by hæmorrhage.

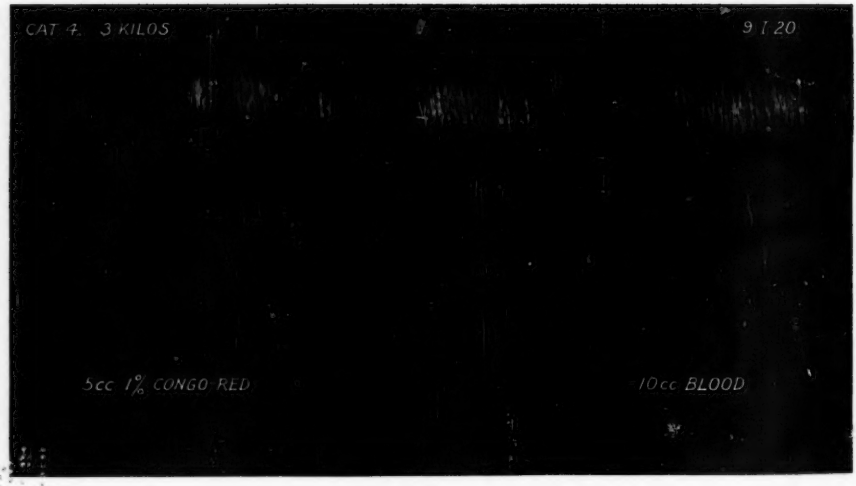
A comparison with the results obtained with vital red shows Congo-red to be very much nearer the true value as in the following analysis:

	Cats. Blood-weight.	Cats. Correction factor.	Dogs. Blood-weight.	Dogs. Correction factor.
Bleeding	4·8 per cent.	× 1·0	7·0 per cent.	× 1·0
Congo-red	5·5 ,,	× 0·85	7·6 ,,	× 0·9
Vital red	6·3 ,,	× 0·78	8·9 ,,	× 0·78

The reason for this is shown in Curve 6, where the rate of disappearance



Tracing 2.



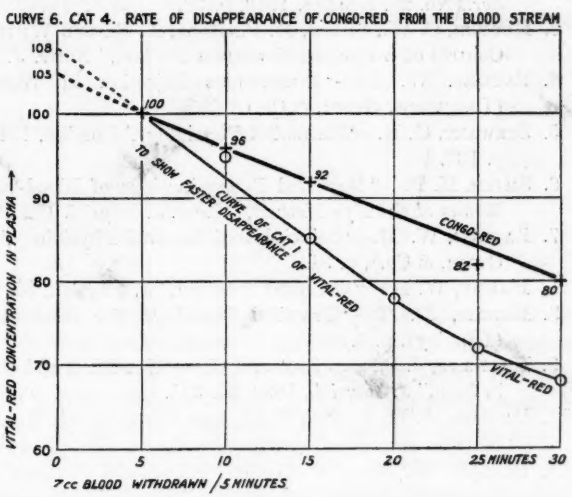
Tracing 3.

of Congo-red (in a cat of 3 kilos.) is seen to be much less rapid than that of vital red (in a cat of 3·8 kilos.).

It appears, however, in the ureters and thoracic duct at about the same time after injection as does vital red:

	Thoracic duct.	Ureters.	
Vital red .	7 min.	11 min.	(Dog 9).
Congo-red .	8 min.	10 min.	(Dog 10).

The physiological action of Congo-red injected in the small doses required for these determinations is not appreciable; even in much larger doses the effect on the blood-pressure, pulse and respirations is only slight, as is shown in the tracings Nos. 2 and 3, in which five times the suggested clinical dose was injected.



So far, then, it appears that Congo-red may prove a better dye for the estimation of the blood-volume in man. Further investigation is being carried out.

CONCLUSIONS.

1. A control method is described for the determination of the blood-volume in animals which depends on removing the maximum amount of Hb. compatible with life whilst keeping the blood-volume constant by the simultaneous injection of gum-saline at the same rate.

2. The vital-red method gives too high a value because the dye leaves the blood-vessels even before it has spread evenly through the circulating fluid. The optimum interval for withdrawal of the blood sample is probably 2–2½ minutes.

3. The correction factor, to convert the vital-red result into the true value, was found to be 0·78 for cats and dogs for a 4-minute sample of dyed plasma. It was as high as 0·9 when a 1-minute sample was taken. It is suggested that the correction factor for man lies between × 0·8 and × 0·9 according to the time of withdrawal of the sample.

4. Congo-red was found to give results nearer the true value, the conversion factor for a 4-minute sample being × 0·9 for dogs.

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CRITICAL REVIEW.

THE ÆTIOLOGY OF INFLUENZA.

(*Concluded from No. 2, p. 126.*)

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THE RELATION OF FILTERABLE VIRUSES TO INFLUENZA.

WITH remarkable unanimity investigators in nearly every country have described an ultramicroscopic virus as the cause of influenza. This unanimity alone carries with it considerable weight, but it must be confessed that the trivial nature of most of the papers and the obvious lack of experience in some of the investigators, compels caution in accepting the claims. This caution will be understood to be all the more desirable when it is realised that the introduction of the invisible virus concept absolves the discoverers from the necessity of producing evidence of a morphologically characteristic microbe.

In Germany W. Kruse (1), in 1914, claimed as the result of one experiment that common colds are due to a filter-passing organism to which he gave the name *Aphanozoa coryzæ*. His assistant suffered from a cold. The nasal secretion diluted with saline was filtered through a Berkefeld filter and several drops of the filtrate were placed into the noses of thirty members of his class. Fifteen of these developed colds, while only one of the thirty-six uninoculated members of the class succumbed. It is obvious that since the assistant and one of the controls had colds this disease must have been epidemic at the time. The action of the filtrate may have been *nil* or it may have acted as a local irritant to the nasal mucous membrane like distilled water and so accounted for the colds. The author produced no evidence of the existence of a living virus in the nasal secretion at all. Exactly the same result might have been produced by placing formalin in the nose of persons exposed to infection. Therefore his attribution of colds to his so-called "*Aphanozoa coryzæ*" is totally unjustified.

In America Foster (23) repeated and extended the work of Kruse. He made filtrates of nasal secretions from acute colds in the same way as Kruse

and dropped these into the anterior nares of ten soldiers. Seven out of these developed "clear-cut and definite symptoms of acute coryza." The clinical histories are given in detail and are too long to quote. There is no doubt that the men suffered from some nasal affection, but in our opinion none could be really described as having ordinary colds. If they had it may still be objected that they were not isolated in any way and were thus exposed to infection; in fact a relative of one developed a cold at the same time. The author next succeeded in growing the virus in Noguchi's medium,* the inocula being obtained from the filtered nasal secretions of eleven cases of natural colds and five cases of experimental colds. Subcultures of these cultures were obtained with ease. In twenty-four hours a positive growth was recognised by the development of an opalescent ring-like cloudiness above the hæmolytic area and around the fragment of kidney. The cloudiness after prolonged staining by Giemsa showed, under the microscope, minute coccoid bodies (illustrated). These bodies are accepted as microbes, the cloudiness in the tubes is evidence of growth, and the cultures applied to eleven further soldiers gave all "unmistakable symptoms of acute coryza."

In 1917 Dold (4), working in a German educational institute in Shanghai, carried out a similar experiment with similar results upon Chinese students, all of whom lived and slept together. He then inoculated forty Chinese hospital patients suffering from minor surgical diseases and all retained in bed. Fifteen other patients in the same ward were used as controls. In this experiment no one became ill. The difference in the results of the two experiments suggests that the students were living under conditions which rendered them liable to colds while the patients were not, and that the inoculation had no special bearing upon the incidence of the disease.

The author then proceeded to grow his invisible virus in Noguchi's medium. In two days growths identical to Foster's were obtained; these were propagated to the fourth generation. In stained preparations nothing could be seen, but under dark-ground illumination numerous minute particles in active movement were observed. This culture placed into the nostrils of three Chinese produced in two increased nasal secretion and slight cough.

The author thus claimed that he had discovered a characteristic virus as motile particles, that these particles were living because they were propagated in culture and that they reproduced their characteristic lesion in man.

On the other hand it will be manifest to anyone who has had experience

* Noguchi's medium consists of unheated ascitic-fluid-broth covered with a layer of paraffin. At the bottom of the tube is placed a fragment of fresh rabbit's kidney. Ascitic fluid is extremely difficult to obtain sterile, in fact it is probably contaminated while still in the peritoneal cavity. These contaminations often do not develop for a number of days, and therefore very special care is necessary to be certain that this medium is sterile. Noguchi's medium is selected by workers upon filter-passers, because, by means of it, Noguchi grew the "globoid bodies" which he advanced as the parasites of poliomyelitis and rabies. In this respect, however, his work is not universally accepted.

of Noguchi's medium that the deductions of these authors must arouse suspicion. These growing opacities are to be observed often enough in uninoculated Noguchi tubes, and, of course, particles demonstrable by prolonged Giemsa staining may be found in most albuminous fluids. Further, the symptoms produced in man were not really very like the natural disease.

We are of opinion that the fact of the presence of these natural opacities makes the whole argument of these authors precarious, and the other evidence adduced is not sufficiently strong to have much weight against this objection. Thus, for the present, we have no reason to believe that common colds are due to filter-passing microbes.

We have reviewed the history of the attempt to associate invisible viruses with colds, because the claims and experimental results adduced are very similar to or in some cases identical to those associating invisible viruses with influenza. Kruse suggested that influenza was due to an invisible virus in 1914 and again in 1918 (5). This suggestion inspired and coloured the later investigations upon influenza, at any rate so far as Germany was concerned.

v. Angerer (6) found by dark-ground illumination "minute particles" in glucose-broth cultures of the filtered blood of rats inoculated with influenza sputum. He made no attempt to demonstrate that these particles were living or capable of producing disease.

Leschke (7) filtered lung-juice or sputum of cases of influenza, and in the incubated filtrate found similar particles in active Brownian movement. The filtrate of sputum inhaled in a spray by the author and several others produced no effect. The experiment was, however, repeated in September, 1918, upon an unstated number of persons and these were all attacked with influenza, but so also were two other persons in contact who had not been sprayed. This author also makes no attempt to show that he is dealing with a living virus, while his experimental transmission of the disease was obviously carried out at a time when influenza was rife among his subjects.

Selter (8) filtered the pooled throat washings of five cases of influenza, and he himself and his assistant inhaled the sprayed filtrate. Next morning he had a "cold," without fever, and a headache. The symptoms were gone in the evening. His assistant next day complained of headache and feeling tired. On the second day she (she was a female assistant) woke with a headache and temperature 37·6° C. In the second night she shivered and sweated. Fejes (9), without foundation, describes this patient as suffering from "several days fever." Thus this author gives no evidence of a living microbe in his material, neither does he reproduce a disease which has any resemblance to influenza.

Fejes himself found the same Brownian particles in ascitic broth cultures of filtered influenza sputum. He carried out inoculations upon four apes, giving each an unstated quantity, and at the same time four other apes were injected as controls with heated filtrate. These remained alive. The first four apes died in from nine to twenty-four days and, at the autopsies, revealed

a picture of "hæmorrhagic sepsis." Hæmorrhages, large and small, were found in all parts of the body. No mention is made of clinical symptoms, microscopic or bacteriological examinations. It is remarked that the filtrates were only used when sterile, thus it may be assumed that the filters used were sometimes defective. This author also produces no evidence that his filtrates contained living organisms of any sort, neither does he appear to have attempted to exclude ordinary microbes as the cause of the "hæmorrhagic sepsis."

Prell (10) did little more than find granules in sections of influenzal lungs stained with iron-hæmotoxylin. These granules he called *Ænigmoplasma influenzæ* and claimed that they were the cause of influenza.

In France the filter-passing concept was supported by Nicolle and Lebailly (11). Seven experiments were carried out upon men and monkeys and in three "positive" results were obtained. The first need not be discussed since it relates to the inoculation of whole influenza sputum under the conjunctiva of a monkey with the production of fatigue, diarrhœa, wasting and depression. Some more of the sputum was diluted, filtered through a Chamberland candle and inoculated subcutaneously into a man: "he fell ill on the same day as the monkey; he presented the classical symptoms of influenza, sudden onset, headache, general depression, oculo-nasal catarrh." The temperature rose to 102° F. In another similar experiment upon man, "the symptoms were those of a slight influenza." The other experiments were negative.

As in the German work, there is no evidence in this of a living virus. Passage experiments were either negative or not attempted. The authors merely demonstrated the presence of a toxic substance in sputum. The same substance might also be present in non-influenzal sputum.

In England, Rose Bradford, Bashford and Wilson (12) claimed to have cultivated a filter-passing organism from a number of diseases, including influenza. Owing, however, to the criticisms of Arkwright (13) these claims appear to have been withdrawn.

Gibson, Bowman and Connor (14), also carried out a large series of experiments with the same objects, but owing to the unfortunate death of one of the workers, the research was left unfinished. They inoculated filtered influenza sputum into Noguchi's medium and observed the growth of a cloud above the fragment of kidney. On prolonged staining this cloud was found to consist of minute "coccoid bodies." These bodies upon being transferred to other Noguchi tubes developed again and were apparently propagated indefinitely. These cultures, inoculated into sundry animals, produced dilatation of the lung capillaries with hæmorrhages of varying extent and exudates into the alveoli or bronchioles. The coccoid bodies were recovered in culture from the kidneys of these animals, and these cultures reproduced the disease. The kidneys of normal animals failed to grow these coccoid bodies.

These experiments were published so shortly that it is a little difficult to criticise them. It is not clear in what way the coccoid bodies differ from the similar bodies which, in spite of their statement to the contrary, are to be observed in uninoculated Noguchi tubes or, indeed, in many albuminous mixtures. They are clearly the same bodies as the "minute particles" of the Germans. Angerer himself (6) admits that hæmoglobin is the best medium for their development. Olsen (15) also was able to manufacture them at 37° C. or 56° C. Again it is not quite clear whether the descriptions of the lesions mentioned above apply to the passage animals or to other animals inoculated with other materials in other experiments. It is a general composite description of the usual results of their work. The only definite statements with regard to the passage experiments are "Its lungs showed some hæmorrhagic areas," and "although the lungs of the animal did not show any typical signs of a marked infection."

The composite picture itself, illustrated by photographs, appears to be based largely upon the results obtained with monkeys, and it is notorious that inflammatory lesions in the lungs of these animals in captivity occur spontaneously.

It is thus seen that as the matter is left the experiments are not conclusive.

The filter-passing work upon influenza has been often repeated but with negative results, and these have usually remained unpublished. However, Lister and Taylor (16) published negative results. Eleven men, who had never had influenza, were removed 500 miles to an island where influenza had not occurred. Nasal washings and sputum from early cases of influenza, both filtered and unfiltered, were introduced into the noses of these men and also into monkeys. In every case the filtered material produced no effect, while two men who received the unfiltered washings developed typical influenza. At the time of the experiment the epidemic had passed.

Wahl, White, and Lyall (17) made extracts from influenzal lungs and sprayed this material, filtered, into the noses and nasopharynges of two volunteers. They remained well.

During the height of the epidemic in November, 1918, one of us (J. McI.) attempted to confirm the existence of filter-passing organisms in influenza.

(1) The filtered nasal washings from two early uncomplicated cases of influenza were inoculated into two *Macacus rhesus* monkeys, four rabbits and two guinea-pigs; 0·1 c.c. was given subconjunctivally and 5 c.c. subcutaneously. There was no effect.

(2) The filtered throat washings from three acute early cases were inoculated in the same way (but 8·0 c.c. subcutaneously) into a *Macacus rhesus* and a *Hussar* monkey. There was no effect.

(3) The filtered lung-juice from necropsies on three fatal cases was inoculated, as in the first series, into a *Macacus rhesus*, a dog-faced monkey, and two rabbits. The dog-faced monkey showed a result as follows: "This animal,

ten days later, developed a running at the nose and a temperature of 103·2° F., but an uninoculated monkey in the next cage had a bad cold. The animal got gradually weaker and died thirty-four days after the inoculation. A necropsy showed an accumulation of fluid in the pleural and pericardial cavities. The lungs showed no congestion, only a few consolidated areas. The spleen was slightly enlarged and showed a number of small yellow areas. Bacteriological and histological examination revealed a recent septicæmia with miliary abscesses. A passage inoculation was made into a *Macacus rhesus* without result."

We do not look upon this as a case of influenza produced in a monkey, but as a spontaneous disease of non-influenzal origin.

To sum up, the positive results of the filter-passing work upon influenza are unconvincing. The characteristic microbe adduced is indistinguishable from undoubtedly inanimate particles which are found in albuminous fluids. The attempts to demonstrate life in these consist in observing the development of cloudiness in other albuminous fluids after inoculation with the particles. Uninoculated tubes are sometimes said to be unaffected, but we ourselves, when engaged upon other work, have seen such opacities develop spontaneously in Noguchi medium. We have thought that they might be globin from the dissociation of hæmoglobin. Such a dissociation might be accelerated by ferments derived from the inoculum or from the kidney, or might be due merely to the action of heat upon this unstable substance.

Arkwright (13) in his attempts to grow the virus of trench fever and influenza in Noguchi's medium, observed these opacities in inoculated or uninoculated tubes. He had the opportunity of examining the preparations and cultures of Bradford, Bashford and Wilson, and found that the bodies described by them as the virus of influenza and of other diseases were identical to the bodies in uninoculated tubes, and, further, the cultures said by them to be pure were in fact contaminated with ordinary bacteria.

It may be pointed out that these coccoid bodies from Noguchi medium have now been described as the cause of common colds, influenza, trench fever and eleven other diseases.

Under the circumstances it is impossible to accept them as bearing upon the influenza problem.

With regard to the experimental transmission of the disease by means of filtrates, those relating to man are unsatisfactory in that the subjects were not isolated or that the illness produced was not influenza. It cannot be said that the lesions in animals were not those of influenza because the effect of this virus upon animals is unknown.

THE RELATION OF *B. INFLUENZÆ* TO INFLUENZA.

As already indicated, reports on the *incidence of B. influenzæ* in influenza have been highly contradictory. It is clear that in the recent epidemic, complicating infections with pyogenic cocci were very much more numerous than

in Pfeiffer's day, and so much more difficult was it to isolate the minute colonies of *B. influenzæ* on blood-agar. The reports from the earlier stages of the epidemic, therefore, show a general inability to demonstrate the bacillus in more than a small number of cases.

The situation was, however, entirely altered by the re-discovery of the importance of altered as opposed to unaltered blood for the growth of the bacillus. These media have a marked selective action and enormously increase the ease with which *B. influenzæ* may be recovered. Attention should, therefore, only be directed to the work of those authors who used one of these selective media.

In the sputum of uncomplicated cases of influenza Matthews (18) found *B. influenzæ* constantly (12 cases), Eyre and Lowe (19) in 12 out of 14, Fildes, Baker and Thompson (20) in 12 out of 15, and McIntosh (21), in an acute epidemic among nurses, in 8 out of 12. Martin (22) investigated a small outbreak in France and found *B. influenzæ* in 76 per cent. More recently Schorer (23) in 81 cases found the bacillus in 91·5 per cent. On the other hand, Little, Garofalo and Williams (24) failed to find *B. influenzæ* in the exudates from the upper air-passages and used this as an argument against the influenzal nature of the epidemic. The negative results, however, are to be explained on the grounds that they used unsuitable medium (legumen-serum-agar). The numerous negative findings among German workers—Gruber, Kolle, Benda, Friedemann, Kruse, etc.—as reported by Friedberger and Konitzer (25) are probably due to the same cause.

In the sputum of cases complicated with pneumonia the presence of other bacteria tends to dilute or cause a disappearance of *B. influenzæ* to some extent, especially in the epidemic period, but Hammond, Rolland and Shore (26) investigated an epidemic of "purulent bronchitis" during the winter of 1916–17 before the main epidemic started. In a series of 20 cases the bacillus was found in 18 by microscopic and cultural examinations. They therefore considered that *B. influenzæ* was the cause of the epidemic on the grounds that the organism was almost constantly present in the sputum and pus of affected bronchioles, in some cases uncontaminated by any other organism. Shortly after this Abrahams, Hallows, Eyre and French (27) grew *B. influenzæ* from 7 out of 8 cases of purulent bronchitis.

At the height of the epidemic period Eyre and Lowe (19) isolated this bacillus in 12 out of 14 sputa, Fildes, Baker and Thompson (20) in 45 out of 106 cases, McIntosh (21) in 42 out of 69, while Messerschmidt, Hundeshagen and Scheer (28) demonstrated Pfeiffer's bacillus in 48·9 per cent. of the June–July epidemic and in 90 per cent. of the September epidemic. Pfeiffer (29) himself states that in 217 sputa sent with the clinical diagnosis of acute influenza, the bacillus was present in 51 per cent. Dick and Murray (30) found it in 63·2 per cent., Park (31) in 80 per cent., Medalia (32) in a large series of 2279 cases in 76·8 per cent., and Schorer (23) in 93·8 per cent. of

260 cases. The variation in these figures depends very largely upon the number of examinations made in each case and the quality of the specimens of sputum forwarded for routine examination.

In the blood before death *B. influenzæ* is only present on rare occasions. Fleming (33) and Medalia (32) found it a few times, but most workers gave uniformly negative results (Fildes, Baker and Thompson [20], McIntosh [21] and Dick and Murray [30]). Wilson and Steer (34) found the bacillus twelve times in the heart-blood post mortem and similar findings have been recorded by others, but in such cases it is always associated with other bacteria. Isolated records are found of the bacillus in empyemata, pericarditis, meningitis, etc.

At autopsies in the beginning of the influenza epidemic almost the only organisms of importance which were isolated from the lung lesions were streptococci and pneumococci. Much time was spent without obtaining any evidence of the presence of *B. influenzæ*. The introduction of selective media, however, resulted in the demonstration of the bacillus in a large number of cases, as may be seen from the figures given below. Thus, Fildes, Baker and Thomson (20), as also Patterson, Little and Williams (35), record its presence in almost all post-mortem material. Muir and Wilson (36) were successful in 30 fatal cases, and remarked that *B. influenzæ* at least played a very important part in the production of the lesions. Lister (37) isolated *B. influenzæ* 53 times from the lungs of 56 fatal cases in South Africa. Olsen (15) found the bacillus in 75 per cent. of the lungs of 220 post mortems, Tytler, Janes and Dobbin (38) in 60 out of 67 cases, Keegan (39) in 82 per cent., Leichtentritt (40) in 60 per cent. McIntosh in an unpublished series found the bacillus in 40 out of 50 cases, and in a second series in 94 per cent. The negative findings have been recorded particularly by a number of German pathologists whose names have already been noted.

Histological examination has shown that *B. influenzæ* occurs in close relationship to the pathological lesions of the disease. It is found throughout the whole respiratory tract from the tonsils to the alveoli of the lungs. In uncomplicated cases it is present in a purer state the lower the examination is made. In films from the lower end of the trachea or of the pus which may be expressed from the bronchioles the bacillus is often found in a pure state and in large numbers and characteristically enclosed within leucocytes (Dietrich (41), Wolbach (42) and personal observations). Beyond the lymphatic tissues of the trachea and lungs, there is, as a rule, no general invasion of the tissues such as may be the case with streptococci and pneumococci in fatal cases. From time to time, however, *B. influenzæ* is to be found in the heart-blood and in empyema pus, while the serous exudates in the neighbourhood of inflamed tracheal and bronchial glands frequently contain the bacillus.

B. influenzæ has been demonstrated also in the respiratory tract of a variety of other conditions such as measles, whooping-cough, phthisis, etc., but at present the exact frequence is difficult to estimate as the figures given by

different workers vary greatly. In such conditions as measles in which the highest findings have been obtained the differentiation from *B. pertussis* (Bordet) must complicate matters. In trachoma, hæmophilic bacilli indistinguishable from *B. influenzæ* have been found in as high percentage as 50, but in this case it is quite possible that *B. influenzæ* plays an important *rôle* since personal observations have shown that this bacillus is not an uncommon cause of acute purulent conjunctivitis. But estimates based on patients in hospital wards, and especially during an epidemic, tend to be fallacious, owing to the rapidity with which one carrier of the bacillus can infect the remainder of the ward. The same, of course, is true with any of the microbes to be found in the respiratory tract. In the case of hæmolytic streptococci Cole and MacCullum (43) found that in the measles ward the incidence was 56·5 per cent. although on admission the incidence was only 11·4 per cent.

The extent to which *B. influenzæ* is normally present in the nasopharynx of healthy individuals is difficult to estimate, as most of the figures available are based on observations made in an epidemic period. It will, therefore, be necessary to wait for some time until accurate and reliable figures can be obtained. Fildes, Baker and Thompson (20) in November, 1918, examined 71 apparently healthy men and obtained 15 positive cultures—21·1 per cent. Later, Fildes (unpublished observations) examined 76 boys on entering a training establishment and found 18 positive. In February to March, 1919, he made cultures from the throats of 177 healthy men in a barracks and obtained 64 per cent. positive. In a training school for girls, Wadsworth (44) was able to find *B. influenzæ* in only 3 of 461 inmates. Lord, Scott and Nye (45) found bacilli in the throats of 76 per cent. of 34 students; Opie, Freeman, Blake, Small and Rivers (46) in 35 per cent. of soldiers in camp. Pritchett and Stillman (47) found that 43 per cent. of normal individuals in the personnel of a large hospital carried the bacillus.

To sum up, therefore, the question of the incidence of *B. influenzæ* in influenza, there can be no doubt that with a satisfactory technique it can be demonstrated in such a large number of cases that it qualifies for being considered of ætiological importance so far as Koch's first criterion is concerned. Not only is it present in a large proportion of cases but it has a close relationship to the lesions of the disease. It is true that it is also found in a number of conditions which are not influenza and in a large number of normal persons, at any rate during epidemic periods, but as has been pointed out above, these facts do not in themselves in any way militate against it being the cause of influenza.

The association of B. influenzæ with other bacteria.—We have already mentioned that in the pneumonias which complicate influenza *B. influenzæ* is apt to be overgrown by a number of other bacteria. These infections with streptococci and pneumococci were the outstanding feature of the change from a relatively benign disease which was observed during the height of the

epidemic. The pathological picture changed to one of an acute septic broncho-pneumonia and in the lesions were found innumerable cocci and but few *B. influenzæ*. From time to time these septic microbes were described as the cause of influenza, but mostly they were recognised as secondary infections in persons intoxicated with the influenza virus.

It was characteristic that the nature of the infecting organism was different in different localities, or even changed in the same locality.

At one period they might be pneumococci, at another hæmolytic streptococci, and at yet another Gram-negative cocci. In this country streptococci were more frequent than pneumococci, although in America the opposite seemed to be more often the case.

Thus Fildes, Baker and Thompson (20) found hæmolytic streptococci in the majority of their post-mortems, Opie, Freeman, Blake, Small and Rivers (46) found that the incidence varied from 4 to 14 per cent. according to the type of case. Cole and MacCallum (43) also found hæmolytic streptococci in a large number of cases in an epidemic of pneumonia, and frequently in association with *B. influenzæ*.

Pneumococci were found to be present by Opie and his co-workers in from 53·6 to 69·8 per cent., and by Small and Stangl (48) in 72·7 to 84·4 per cent. McIntosh (unpublished) found pneumococci in about 30 per cent. and streptococci in 30 to 37 per cent., about one-half of which were of the hæmolytic variety.

Staphylococci were not so infrequent as would be imagined; in some instances they appeared to take on epidemic form, and it was suggested (Patrick [49]) that they were of some ætiological importance in epidemics of broncho-pneumonia. Fletcher (50) demonstrated the presence of meningococci in large numbers in association with *B. influenzæ* in the lungs of eleven fatal influenzal broncho-pneumonia cases.

From time to time other organisms such as the pneumo-bacillus, pleomorphic streptococci, *B. proteus*, etc. were found in considerable numbers.

Thus an association or a superimposed infection of various other bacteria, which resulted in an increased virulence of the disease, was a feature of the recent epidemic.

Attempts to demonstrate directly a *pathogenicity of B. influenzæ* to man have been almost uniformly unsuccessful. Lister and Taylor (16) introduced living suspensions of this bacillus and of two varieties of cocci into the noses and mouths of 9 men, and only 1 of those who received *B. influenzæ* fell ill. The authors would not, however, ascribe this illness to influenza. Rosenau (51) treated 19 men with thirteen strains in suspension with negative results, and Wahl, White and Lyall (17) were equally unsuccessful with 5 men.

Although, therefore, there is no satisfactory evidence that *B. influenzæ* is pathogenic to man so far as the inoculation of cultures goes, nevertheless the

constant occurrence of anti-bodies to this bacillus in the serum of patients makes it clear that it has a definite pathogenic action, which cannot, however, be reproduced. The presence of the anti-bodies may be taken as evidence that the bacillus is a pathogenic bacillus such as might be the cause of influenza, but not that the characteristic lesions of this disease are due to this pathogenic bacillus.

The pathogenic action of *B. influenzæ* upon animals has been studied by many. In general it may be said that the effect of suspensions of bacilli from pure slope cultures, even in massive doses, is remarkably slight on laboratory animals. Introduced into the peritoneal cavity, a certain amount of inflammation is found depending upon the strain, but the bacteria rapidly disappear, and, as a rule, no permanent effect is produced.

On the other hand, liquid cultures very often exhibit marked pathogenic action. The success of these experiments depends upon the selection of a strain with the required effect, since many have little toxicity even under these conditions and the toxicity cannot, apparently, be increased by passage.

When introduced into the peritoneal cavity of the guinea-pig a marked peritonitis may occur with œdema of the belly wall, diarrhœa and prostration. The suprarenals are red and congested, but no constant effect is observed in the lungs.

Blake and Cecil (52) by means of liquid cultures which had been passed through a series of mice and then recovered from the peritoneal cavity of monkeys, were able to produce in monkeys an infection of the upper respiratory tract which might be complicated by acute sinusitis, tracheo-bronchitis and broncho-pneumonia.

In the first series 12 monkeys were inoculated by swabbing the naso-pharynx with a swab dipped in the culture or by directly dropping the liquid culture into the nose by means of a pipette (1 c.c.). In the animals successfully inoculated there was a rise of temperature with symptoms of coryza, blinking of the eyes, rubbing of nose, etc. The infection as a rule lasted from three to five days. Complications set in in from twenty-four to forty-eight hours, and were usually marked by a cough and scanty muco-purulent secretion. Complications which developed were 5 cases of sinusitis of the antrum of Highmore and 2 of broncho-pneumonia. Necropsies of the two latter showed extensive hæmorrhages, peribronchial areas of consolidation with exudate of leucocytes, mononuclear and epithelial cells, together with an infiltration and thickening of the alveolar walls. *B. influenzæ* was obtained from both lungs in pure culture.

In the second series 10 monkeys were inoculated with 1 to 5 c.c. of the culture intratracheally through the skin by means of a syringe. Of the 10 monkeys thus inoculated 2 developed tracheo-bronchitis and 7 pneumonia. Necropsies of the pneumonic monkeys showed a widespread patchy pneumonia, with extensive hæmorrhages, emphysema, œdema and slight cellular infiltra-

tion. In some of the animals the type of pneumonia was rather different in that it was much more general and there was extensive bronchitis. The authors considered that the pathological picture in these lungs was identical with that observed in influenza in man.

The cause of the toxicity of liquid cultures was shown by Parker (53, 54) to be due to the presence of a bacterial toxin. If a liquid culture of a suitable strain was filtered and injected intravenously into rabbits the animal would die in one to two hours. By preparation of animals with this toxin it was possible to produce an anti-toxin which had a certain degree of protective action against the toxin.

Similar results were obtained by Ferry and Houghton (55). Huntoon and Hannum (56) prepared an endo-toxin by grinding the desiccated bacteria and extracting with saline. This had a toxic action on mice and other animals, and, according to the authors, by whatever route it was introduced it caused acute congestion of the lungs and hæmorrhagic exudates into the pleural cavities.

McIntosh (unpublished) found that for rabbits the toxicity of the filtrates of these cultures was demonstrated in quantities of 5 to 6 c.c. or less. In some cases death followed immediately after intravenous injection, in others the animal became ill and lay in a collapsed condition, death usually occurring next day. In these animals a post-mortem examination revealed numerous subcutaneous hæmorrhages and characteristic lung changes.

In the guinea-pig the average intravenous lethal dose was 2 or 3 c.c., although with some cultures it was as low as 0·5 c.c. or less.

Perhaps the most suggestive syndrome was that observed in guinea-pigs when death occurred in four or five hours after the injection. Immediately following the injection the animals became collapsed, the hair stood on end, and they tended to crouch in a corner of the cage. About an hour later the temperature was found to be subnormal, there was a discharge of frothy mucous at the nose, and slight diarrhœa. The pathological changes observed were often quite striking, in fact it was surprising that such extensive changes could have occurred so quickly. This was particularly true of those animals which died in from twenty-four to forty-eight hours. The changes were identical with those observed in rabbits, only they were more intense. The lungs were, as a rule, voluminous and did not retract fully when the chest was opened. On the surface were to be seen slightly sunken red areas of collapse alternating with paler areas of emphysema. In some, definite hæmorrhagic areas were observed, and these at times involved a whole lobe of the lung. On cutting into the lungs, blood-stained, frothy mucus escaped in considerable amount.

Microscopically the affected areas showed a typical picture of broncho-pneumonia. The bronchioles were plugged with desquamated and proliferated epithelium, together with a few leucocytes. There were hæmorrhages into some bronchioles. The bronchial walls and the peribronchial interstitial

tissues were infiltrated and contained small hæmorrhages. Groups of alveoli were filled with desquamated epithelium and leucocytes. Many alveoli contained hæmorrhages and coagulated albuminous fluid.

The supra-renals were œdematous and frequently the seat of numerous hæmorrhages. The kidneys showed a varying amount of parenchymatous degeneration with actual necrosis of the epithelium in places. The intestines were usually congested and distended with gas, and in places showed subperitoneal hæmorrhages.

These lesions appeared to be characteristic of *B. influenzæ*, and were not caused by the injection of filtrates from cultures of streptococci or pneumococci.

Thus, by applying *B. influenzæ* to animals in a form which would not be permissible to man, a constant pathogenic action may be demonstrated in many strains, and the outstanding feature of the lesions produced is a broncho-pneumonia.

The pathogenic action of *B. influenzæ* in association with other bacteria has been studied. Huntoon and Hannum (56) found that the mixed toxins of *B. influenzæ* and streptococcus were more toxic than that of *B. influenzæ* alone, and that if animals were prepared with *B. influenzæ* toxin they might succumb to a spontaneous streptococcal or pneumococcal pneumonia. Yanagisawa (57) also found that the presence of *B. influenzæ* increased the pathogenicity of streptococci and pneumococci.

These results may account for the frequence of virulent secondary infections in influenza.

To sum up the evidence of pathogenicity of *B. influenzæ* to animals, it may be accepted as established that fairly constant lesions may be produced by certain cultures applied in particular ways to susceptible animals. The most characteristic lesion to be observed is a broncho-pneumonia.

The precise relationship of these experimental lesions in animals to influenza in man is difficult to define, because the true influenza syndrome is not yet clearly recognised, and the disease is very little susceptible to experimental transmission. Very extensive efforts have been made in America to transmit influenza to man experimentally (Rosenau [51]). Mixed nasal and throat washings from patients were sprayed into the noses and throats of 10 volunteers or even directly transferred on a swab; 10 c.c. of blood from influenza patients were also inoculated into 10 men; and, finally, 10 men deeply inhaled the breath and received the coughs of 10 patients. None of these men developed any illness.

On the other hand Lister and Taylor (16), using nasal washings and sputum of cases, described two positive results. They introduced this material into the throats and nasal cavities of men and monkeys, and two men out of five developed influenza, although the same material, filtered, produced no effect.

In the absence of precise knowledge, then, we may define influenza as an infective illness which is extremely difficult to transmit wilfully to another

person. Its characteristic lesion is an inflammation of the respiratory tract, especially of the trachea and bronchioles in more advanced cases. Depression and intoxication are also conspicuous. Finally the disease is liable to acute outbursts during which the benign character changes, and then it becomes associated with numerous septic bacteria.

The disease produced in animals by *B. influenzæ* is also only brought about with difficulty under conditions which ensure the presence of a large amount of toxin. The bacillus itself is nearly ineffective. The disease when produced is essentially an inflammation of the respiratory tract which has a very close resemblance to that observed in many cases of influenza. The intoxication of the animals is always well marked. Lastly there is evidence that an intoxicated animal is more susceptible to the attacks of other bacteria than a normal animal, and may even spontaneously develop pneumonia.

Thus, we have a characteristic living organism which is present in the lesions of influenza and has a pathogenic action for man. Pure cultures of this bacillus are capable of producing in animals a condition which has important points in common with influenza in man, and it is only the indefinite nature of the essential lesions of influenza which causes hesitation in accepting the experimental disease in animals as the same as the natural disease in man.

If this relationship is accepted, then the criteria by which a bacterium should be judged the cause of a disease are fulfilled.

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